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Abstract: Glioblastoma (WHO grade IV glioma) is the most common malignant primary brain tumor in adults. Survival has remained largely static for decades, despite significant efforts to develop new effective therapies. Immunotherapy and especially immune checkpoint inhibitors and programmed cell death (PD)-1/PD-L1 inhibitors have transformed the landscape of cancer treatment and improved patient survival in a number of different cancer types. With the exception of few select cases (e.g., patients with Lynch syndrome) the neuro-oncology community is still awaiting evidence that PD-1 blockade can lead to meaningful clinical benefit in glioblastoma. This lack of progress in the field is likely to be due to multiple reasons, including inherent challenges in brain tumor drug development, the blood-brain barrier, the unique immune environment in the brain, the impact of corticosteroids, as well as inter- and intratumoral heterogeneity. Here we critically review the clinical literature, address the unique aspects of glioma immunobiology and potential immunobiological barriers to progress, and contextualize new approaches to increase the efficacy of PD-1/PD-L1 inhibitors in glioblastoma that may identify gaps and testable relevant hypotheses for future basic and clinical research and to provide a novel perspective to further stimulate preclinical and clinical research to ultimately help patients with glioma, including glioblastoma, which is arguably one of the greatest areas of unmet need in cancer. Moving forward, we need to build on our existing knowledge by conducting further fundamental glioma immunobiology research in parallel with innovative and methodologically sound clinical trials.

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PD-1 inhibitors: Do they have a future in the treatment of glioblastoma?

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Abstract

Glioblastoma (WHO grade IV glioma) is the most common malignant primary brain tumor in adults. Survival has remained largely static for decades, despite significant efforts to develop new effective therapies. Immunotherapy, and especially immune checkpoint inhibitors and programmed death (PD)-1/PD-L1 inhibitors have transformed the landscape of cancer treatment and improved patient survival in a number of different cancer types. With the exception of few select cases (e.g., patients with Lynch syndrome) the neuro-oncology community is still awaiting evidence that PD-1 blockade can lead to meaningful clinical benefit in glioblastoma. This lack of progress in the field is likely to be due to multiple reasons, including inherent challenges in brain tumor drug development, the blood-brain barrier, the unique immune environment in the brain, the impact of corticosteroids, as well as inter- and intra-tumoral heterogeneity.

Here we critically review the clinical literature, address the unique aspects of glioma immunobiology and potential immunobiological barriers to progress, and contextualize new approaches to increase the efficacy of PD-1/PD-L1 inhibitors in glioblastoma that may further stimulate pre-clinical and clinical research to ultimately help patients with glioma, including glioblastoma, arguably one of the greatest areas of unmet need in cancer. Moving forward, we need to build on our existing knowledge by conducting further fundamental glioma immunobiology research in parallel with innovative and methodologically sound clinical trials.

Introduction

Glioblastoma (GBM; grade IV glioma) is the most common and aggressive type of malignant primary brain tumor. The standard of care for glioblastoma, maximal safe surgical resection followed by radiotherapy plus temozolomide and adjuvant temozolomide, achieves a median overall survival of 15 months¹⁻³. Despite recent advances in multi-modality therapy for glioblastoma, incorporating surgery, radiotherapy, systemic therapy, tumor treating fields, and supportive care, median survival remains static at about 15 months, and long-term survival is rare¹. Two of three patients have an unmethylated O⁶-methylguanine-DNA methyltransferase (*MGMT*) promotor that renders them less likely to benefit from DNA alkylating agents such as temozolomide or nitrosoureas, and their median survival is even shorter, at about 12 months⁴.

Cancer escapes immunosurveillance via several mechanisms, including activation of immune checkpoint pathways that suppress anti-tumor immune responses. Immune checkpoint inhibitors reinstitute the immune response by disrupting co-inhibitory signaling pathways aiming to eliminate tumor cells. Immune checkpoint blockade has transformed the landscape in several cancers, including cancers with brain metastases⁵. The main immune checkpoints that have been successfully targeted with monoclonal antibodies are programmed death-1 (PD-1), PD-ligand 1 (PD-L1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4). Immunotherapy, especially with PD-1/PD-L1 inhibitors, has transformed the management of many cancers, and consequently there has been considerable investigation and research into immune-based therapeutic approaches for glioma, and specifically GBM. However, to date these therapies have not demonstrated a major benefit for these patients. Here, we critically appraise the preclinical and clinical literature to provide a novel perspective on how to make progress in this area of unmet need.

Clinical Trials Targeting PD-1/PD-L1 in GBM

Several phase I/II trials in recurrent and newly diagnosed glioblastoma are evaluating the anti-PD-1 agents nivolumab, pembrolizumab, cemiplimab or the anti-PD-L1 antibodies atezolizumab, avelumab, durvalumab, and other PD-1/PD-L1 inhibitors alone or in combination with other therapies.

Combinations with standard treatment are often developed to test specific synergy hypotheses, such as simultaneous PD-1-inhibition and anti-angiogenic therapy (bevacizumab) and/or radiotherapy. Examples of this approach include a phase II trial evaluating the anti-PD-L1

antibody durvalumab in five different cohorts of patients ⁶, including combinations with radiotherapy for newly diagnosed glioblastoma or with bevacizumab for recurrent glioblastoma. Numerous studies of immune checkpoint inhibitors, in newly diagnosed and recurrent glioblastoma are ongoing (Table 1) and several studies including phase III studies with nivolumab have been reported (Table 2). None of these studies reported findings that could lead to approval of these agents.

Trials have also been conducted with neoadjuvant (pre-operative) administration of PD-1 inhibitors to induce a more pronounced immune response than with the usual post-resection approach (Table 3). In addition to the effect of perioperative corticosteroid administration, surgery itself may dampen the immune response, with suppression of cellular immunity as one of the host responses to surgical stress⁷.

In summary, the phase II and III studies in GBM with nivolumab have not demonstrated a meaningful benefit. No other phase III studies with PD-1/PD-L1 inhibitors are planned or have been conducted.

Why are PD-1/PD-L1 Inhibitors Ineffective in GBM?

Challenges with pre-clinical and clinical trial design paradigms

Evaluating immunotherapy relies on pre-clinical models to identify targets, conduct mechanistic studies, and to optimize delivery and pharmacokinetic aspects. These models have helped develop the CTLA-4 and PD-L1/PD-1 inhibitors ^{8,9}. GBM immune biology may not be well reflected in the models that are frequently utilized. A commonly used animal model is the GL261 glioma, which is moderately immunogenic and expresses clonotypic, homogeneous, and robust levels of PD-L1, which is not the case human gliomas ¹⁰. Pre-clinical models are developed by implanting cancer cell lines either in the flank or in the brain of mice. Tumors that are successfully implanted rarely represent the GBM immune complexity seen in humans. Common pre-clinical models are relatively easy to evaluate as subcutaneous implants. Mice can be engineered to approximate, but not completely represent, human GBM. Knocking out suppressor genes or induction of somatic mutations can result in *de novo* tumors, but they do not fully represent the accumulation of genetic aberrations that develop in human GBM. The immune system is dependent on the tumor microenvironment (TME), which is challenging to reproduce in animal models.

Humanized mice models using both tumor and immune systems that are patient-specific are an improvement to study immunotherapies but they are limited by cost, low engraftment rates and slow tumor growth.

The scarcity of representative pre-clinical models has led to the initiation of clinical trials evaluating immunotherapies that have shown promise in other cancers without definitive pre-clinical rationale in GBM. Clinical trials are often initiated with uncontrolled, open label (unblinded), non-randomized, small sample size trials that have inherent selection and other biases. This in turn will have a high type I error rate, with ineffective therapies showing false positive results that may surreptitiously build the case to start larger studies. Large studies minimize risk of bias and false positive results will be less likely to be reproduced. Unfortunately, the true effect size or lack of activity only becomes apparent after hundreds of patients have participated and millions of research dollars have been invested over the course of several years.

Another factor making interpretation of preclinical findings difficult is that there is never a surgical resection of a mouse engrafted with syngeneic glioma cells, which does not recapitulate the large number of GBM patients that undergo surgical resection and are enrolled in clinical trials evaluating PD-1 inhibitors.

Zhai *et al*, demonstrate the use of humanized mice partially reconstituted with a human immune system and intracranially-engrafted with human glioblastoma. The authors demonstrate that the tumor-infiltrating T cells increase immunosuppression in glioblastoma. The take home message from this preclinical study is that it is not sufficient to simply enhance T cell infiltration, but if successful, one must then deal with the consequence of the T cell-dependent increase of glioblastoma-induced immune suppression.

There are other barriers to translating pre-clinical ideas to human trials. For example, immune priming, activation, and memory responses may require sequencing of therapies that are challenging to evaluate using the traditional clinical trial paradigms. Another challenge in glioblastoma is the inability to collect biopsies at different time-points as it is usually done in other solid tumors (at baseline before the immunotherapy, after a few weeks of treatment, and often at recurrence). This is one of the differences in glioma drug development as compared with other cancers.

Challenges with immunotherapy combination studies

Trials need rational combinations backed by plausible pre-clinically tested hypotheses. Integration of pre-clinical and clinical studies to evaluate promising therapies in pre-clinical models with a “bench to bedside and back to bench” approach may detect early signals or declare ineffective combinations in a more rational manner. Combinations, which may include a therapy that has single-agent activity, are challenging to assess in phase I trials due to factors such as patient heterogeneity, selection bias, and lack of randomization. Newer methods of drug development such as randomization into phase I trials may need to be explored ^{11,12}.

Unlike chemotherapy, effective combination immunotherapies may include agents that are unlikely to independently induce a radiologic response. These combinations may not require individual agent activity. Some of the therapies in the combination may have an effect only by sensitizing to a second agent (e.g., the CD40 agonistic antibodies).

Immunosuppression in GBM

Patients with GBM are known to have significant deficits in cell-mediated immunity within the lymphocyte population, and specifically the CD4 cells.

Tumor-infiltrating lymphocytes

Tumors can be defined depending on immune infiltration and are classified as immune desert, immune-excluded, and hot tumors, depending on the presence of tumor-infiltrating lymphocytes (TILs) in tumor specimens ¹³. A tumor that is characterized as an immune desert can arise from immune ignorance, tolerance, or absence of T-cell priming or activation ¹³. In some studies, the numbers of TILs suggest a positive correlation with better prognosis ¹⁴⁻¹⁶. While the presence of lymphocytes in a tumor sample may represent immune engagement, these lymphocytes might be exhausted in GBM and thus may not yield a favorable immune effect. Subsets of TILs may differ, as shown in a study where the level of CD8⁺ T-cells and tumor grade were inversely correlated, while the level of CD4⁺ T-cells and tumor grade were positively correlated ¹⁷. TILs or CD8⁺ T-cell infiltrates have been proposed as a surrogate for the presence of antigen, immune activation, and trafficking to the TME. They are positively correlated with response to therapy in other tumors, particularly melanoma ¹⁸ and lung cancer ¹⁹. Their exact role in gliomas requires further investigation.

T-regulatory cells (Tregs) are a highly diverse and plastic subset of CD4 T-cells and are important in immune tolerance. The thymus-derived natural Tregs (nTregs), characterized by high constitutive expression of FoxP3, cause contact-dependent cytokine-independent immunosuppression through CTLA-4 and PD-L1 pathways ²⁰.

T-cell exhaustion

A study of T-cell exhaustion in GBM in TILs and peripheral blood lymphocytes isolated from 21 patients evaluated multiple immune checkpoint markers, including PD-1, CTLA-4, and TIM-3, and reported upregulation of multiple immune checkpoints. The T-cell transcriptional profile approximated virus-induced exhaustion ²¹. Another study that reported reduced absolute CD4 counts in 20 patients with GBM also observed that the fraction containing CD4⁺CD25⁺FoxP3⁺CD45RO⁺ T-cells was increased compared to healthy controls ²². T-cells from these patients demonstrated anergy or secreted Th2 polarizing cytokines on stimulation. Removal of the Treg population reversed this cytokine signature. Treg removal eliminated T-cell proliferative defects and restored Th2 cytokine shifts, where T-cells from patients with glioblastoma could function *in vitro* at levels equivalent to those of healthy controls.

Another group used glioma samples to demonstrate that the nTreg population is predominant in glioma with high FoxP3 expression ²³. Thymectomy appeared to help reduce Treg levels in the GL261 mouse cell line injected intracranially ²⁴. An analogous approach to restore T-cell function is currently being explored in a trial of lymphodepletion in combination with PD-1 inhibition (NCT02664363) ²⁵.

One study found no correlation with levels of Tregs and prognosis, suggesting that gliomas may also mediate immunosuppression through other mechanisms ²⁶. Some cancers employ loss or downregulation of class I MHC to evade immune destruction ²⁷. PD-L1/PD-1 inhibitor activity enables an endogenous immune response that relies on previously primed CD8⁺ T-cells that specifically recognize cancer cells. These immune cells can either be re-invigorated or expanded by PD-L1/PD-1 inhibition, leading to recognition and targeting of cancer ²⁸.

Immune desert tumors

Immune desert cancers like GBM, hormone receptor positive breast cancer, and prostate cancer respond poorly to immune checkpoint inhibitors ²⁹⁻³¹ for a number of potential reasons,

including lack of immune infiltration and impaired antigen presentation (low MHC class I) and may have high tumor cell proliferation.

Distinct mechanisms associated with the immune desert tumors include tumor-intrinsic WNT/ β -catenin signaling that may reduce the chemokine gradient required for recruiting CD103⁺ dendritic cells (including CXCL1, CXCL2, and CCL4)³². This impacts early T-cell priming and infiltration into the TME. Rapidly proliferating immune desert tumors alter the metabolic conditions in the TME to create a hostile environment for the function and proliferation of T-cells³³. T-cells depend on similar glycolytic pathways as tumor cells for survival³⁴. Lactate production, presence of an acidic TME, and lipogenesis may alter the immunometabolism of T-cells, affecting TCR engagement, T effector activation, differentiation, and proliferation, ultimately resulting in reduction or absence of TILs³³.

Immune ignorance is also associated with the presence of a stem cell-like phenotype in tumors (“stemness”)³⁵. Stemness correlates with higher intratumoral heterogeneity, possibly by protecting antigenic clones from elimination by the immune system. Stemness is associated with cell intrinsic suppression of endogenous retroviruses, type I interferon signaling, and increased expression of multiple immunosuppressive pathways, and may provide a mechanistic link between antigenicity, intratumoral heterogeneity, and immune suppression across cancers.

The TGF- β pathway

The transforming growth factor- β (TGF- β) pathway is known to be a driver of glioma tumorigenicity and immunosuppression. TGF- β secretion is also one of the characteristics of immune desert tumors, in addition to lack of TILs and the presence of myeloid-derived suppressor cells and VEGF-associated tumor angiogenesis^{36,37}. The first description of TGF- β isoform 2 was as a glioblastoma-derived T-cell suppressor factor, measured in GBM cell lines and patient serum³⁸. TGF β was noted to suppress T-cell proliferation through IL-2-dependent and independent pathways. The TGF- β receptor is highly expressed on glioma cells and RNA silencing of TGF- β reduces glioma proliferation, migration, and invasiveness. A clinical trial (n=150) that treated GBM patients with lomustine with or without galunisertib, a TGF- β receptor inhibitor, failed to demonstrate improved overall survival relative to placebo³⁹. New approaches to targeting TGF- β together with PD-L1 are ongoing⁴⁰.

The immunosuppressive effect of corticosteroids

GBM patients typically receive systemic corticosteroid therapy immediately before and after a craniotomy. Steroids modulate the immune system, causing lymphopenia, including reducing the absolute number of T-cells ⁴¹. Corticosteroids are also routinely used to control vasogenic edema in gliomas, as well as for the treatment of a variety of autoimmune diseases, immune-related adverse events triggered by checkpoint inhibition, or cytokine release syndrome. It is very common for GBM patient to have received, or to continue to receive, relatively high doses of corticosteroids, which may diminish T-cell activity, including that associated with immune checkpoint inhibitors.

There are data to support the hypothesis that systemic corticosteroids used to treat immune-related adverse events may not compromise the therapeutic benefit of immune checkpoint inhibitor therapy ⁴². However, it is challenging to ascertain whether benefit was achieved before or after steroids were given. Individuals who develop immune-related adverse events may have more engaged immune systems ⁴³. This creates an inherent bias, especially in retrospective analyses. Assessment of the impact of steroids on immunotherapy will also need to take the timing of steroid administration into account. While corticosteroids may not completely remove the anti-cancer immune response, they can potentially attenuate it. This might be acceptable in an immune dominant tumor environment (such as melanoma), but this effect may be more significant in immune desert tumors such as GBM.

Other unclear aspects include the effects of the type, timing, and overall dose of corticosteroids on response to immunotherapies. These questions are important but difficult to answer in clinical trials.

Macrophages, Microglia and Myeloid-Derived Suppressor Cells (MDSCs)

Microglia and non-parenchymal macrophages in the brain are mononuclear phagocytes that are increasingly recognized to be important in diseases of the brain. Peripheral immune cells can infiltrate the CNS in certain conditions, but microglial precursors migrate to and reside in the brain parenchyma. Brain resident macrophages and microglia are essential part of the innate immune system against various insults. Parenchymal microglial cells and recruited monocyte-derived macrophages from the periphery exhibit disease-specific phenotypic characteristics and are subpopulations based on their molecular signatures. An abundance of myeloid over lymphoid lineage cells during CNS pathology, including gliomas, is a feature of brain immunity as compared to extra-CNS immunity⁴⁴.

Macrophages can be divided into M1 type and M2 type macrophages: M1-type macrophages have anti-tumor features and secrete pro-inflammatory factors such as IL-6, IL-8 and TNF- α , whereas M2-type macrophages have tumor-promoting features and secrete anti-inflammatory factors such as IL-10 and TGF- β , promoting tumor cell growth and migration⁴⁵.

MDSCs play a major role on tumor microenvironment immunosuppression in blood and tumors from glioblastoma patients. MDSC subsets differentially drive immune suppression in a sex-specific manner. monocytic (mMDSC) versus granulocytic (gMDSC) subsets have different contributions⁴⁶. A mouse study showed that mMDSCs were enriched in the male tumors, while gMDSCs were elevated in blood of females⁴⁶. Only female mice had extended survival with depletion of gMDSCs⁴⁶. mMDSCs could be targeted with anti-proliferative agents in male preclinical models, while IL-1b blockade in females inhibit the gMDSC function. In this study, patient data showed that proliferating mMDSCs were predominant in male tumors. A high gMDSC/IL-1b gene signature correlated with worse prognosis of the female patients.

PD-L1 is expressed not only by glioblastoma cells but also detected in extracellular vesicles (EVs). GBM EV-mediate immunosuppression, by inducing immunosuppressive monocytes rather than inhibition of T-cells⁴⁷. PD-L1 expression is important for the induction of specific immunosuppressive monocyte populations but immunosuppression through EVs are complex and not limited to PD-L1⁴⁷.

A recent study reported that resistance to immune checkpoint blockade that is driven by macrophages, is established by CD4 T cell suppression and T_{reg} expansion in the tumor microenvironment via the PD-L1/PD-1/CD80 axis. This study also reported heterogeneity of response in syngeneic tumors and suggest that targeting PD-L1-expressing tumor-associated macrophages might overcome resistance to immune checkpoint blockade.

There are ongoing efforts to exploit these observations therapeutically, including combinations of macrophage/monocytes modulation with immune checkpoint blockade.

Immune Biomarkers

PD-L1 immunohistochemistry

Tumors with high expression of PD-L1 or those with increased tumor-infiltrating immune cells might be considered inflamed and theoretically may respond to PD-L1 and PD-1 inhibitors. While this true to a degree in melanoma, lung, and some other cancers, it has not been demonstrated in gliomas. Tissue testing of PD-L1 expression in general, but also in glioma, has been challenging, with various antibodies and techniques employing variable cut points for positivity ⁴⁸. In one study, about 61% of gliomas had at least 1% of tumor cells expressing PD-L1 ¹⁰, with 38% of gliomas exhibiting at least 5% PD-L1 expression. PD-1, PD-L1, and TIL appear to be positively correlated with tumor grade in all gliomas, and levels of expression are higher in GBM ⁴⁹. In other cancers, responses can be identified in cohorts that do not express PD-1 or PD-L1 ⁵⁰. It is also uncertain whether expression of PD-1 and/or PD-L1 on infiltrating immune and myeloid cells should be considered ⁵¹.

The capacity to present antigens, such as the expression of MHC class I on the surface of tumor cells and an intact HLA, is also essential, but difficult to assess for clinical purposes. Expression of the gene encoding PD-L1 increases post transcriptionally in human glioma after loss of phosphatase and tensin homolog (PTEN) and activation of the phosphatidylinositol-3-OH kinase (PI(3)K) pathway. In one study specimens from glioblastoma patients had levels of PD-L1 protein that correlated with PTEN loss, and tumor-specific T cells lysed human glioma targets expressing wild-type PTEN more effectively than those expressing mutant PTEN. This may suggest a mechanism linking loss of the tumor suppressor PTEN with immunoresistance, mediated in part by PD-L1⁵².

Tumor mutational burden

In melanoma, there is a positive correlation between the median frequency of somatic mutations in tumors and overall response to anti-PD-1 therapies ⁵³. A higher mutational load in tumors may result in more tumor antigens, increasing immunogenicity ⁵³. Higher mutational load in tumors has also been associated with increased survival and long-term benefit from immunotherapy ⁵⁴. Further, RNA sequencing of lung, ovarian, breast, colorectal, brain, and renal cancers has shown that tumor immunogenic mutation load was associated with survival ⁵⁵. Mutational load is thought to be a surrogate marker of neoantigen expression. Recognition of neoantigens is a major factor in the activity of immunotherapies ⁵⁶; and therefore neoantigen load may be a useful biomarker to facilitate the development of therapeutic approaches that selectively enhance T-cell reactivity and may translate to a higher benefit from checkpoint blockade. An increase in mutations as we age is well documented, not only in GBM, but in cancer in general ^{57,58}. This trend can only be seen in exome-wide analysis, not in the commercial next generation sequencing panels that identify genes that are frequently mutated as part of the cancer process ⁵⁹. To test this hypothesis, a phase II trial is currently randomizing 102 elderly GBM patients (≥ 65 years) in a 2:1 fashion to short course radiotherapy followed by temozolomide with or without nivolumab ⁶⁰.

However, techniques and cut points for defining mutational load are not harmonized, so other more easily measured indices have been proposed, such as determining mutations in the exonuclease domain of polymerase E that lead to hypermutation and neoantigen load ⁶¹. Individuals with germline Lynch syndrome mismatch repair (MMR) defects have long been recognized to be at increased risk of central nervous system (CNS) tumors ^{62,63}.

Figure 1

Nonetheless, tumors with very high TMB, of which tumors with microsatellite instability (MSI) represent the highest fraction of the TMB continuum, respond favorably to PD blockade and this has led to the approval of pembrolizumab in any MSI-high tumors, regardless of the organ of origin ⁶⁴. Indeed, patients with biallelic dMMR in glioblastoma have responded to nivolumab ⁶⁵.

A small percentage of newly diagnosed tumors and a larger proportion of tumors during and after standard therapy with radiation and alkylating agents may have DNA repair defects of various types, including a “hypermutator” phenotype that has also been described in GBM specimens with MSH6 mutations ⁶⁶. Another subset of GBMs with a potential hypermutator

phenotype are lower-grade gliomas that recur after treatment with alkylator therapy ⁶⁷. Theoretically, this may make these tumors responsive to immune checkpoint blockade ⁶⁸.

Touat *et al*⁶⁹ analyzed molecular determinants of mutational burden and signatures in 10,294 gliomas. Two main pathways to hypermutation were described, one associated with constitutional defects in DNA polymerase and mismatch repair (MMR) genes, and a more common post-treatment pathway, associated with acquired resistance driven by MMR defects in chemotherapy-sensitive gliomas that relapse following temozolomide.

Experimentally, mutational signature of post-therapy hypermutated gliomas was reintroduced by temozolomide-induced damage in cells with MMR deficiency. MMR-deficient gliomas that lacked prominent T cell infiltrates had extensive intratumoral heterogeneity, poor patient survival, and a low rate of response to PD-1 blockade. Bulk analyses did not detect MSI in MMR-deficient gliomas, but single-cell whole-genome sequencing analysis of post-treatment hypermutated glioma cells identified microsatellite mutations. This study shows that chemotherapy may introduce hypermutated populations without promoting a response to PD-1 inhibitors. Touat *et al* defined high TMB as ≥ 17.0 mut. per Mb. However, the cutoff for TMB is not well defined and response to PD-1 inhibitors in inflamed tumors may differ from that in non-inflamed tumors ⁷⁰. Intratumoral heterogeneity may have a greater impact on immune response and may predict immunotherapy outcomes better than mutational burden ³⁵. All of these complexities make the value of TMB as a biomarker questionable, with respect to highly heterogeneous tumors such as GBM, even in those with MMR defects.

There have been no prospective studies in gliomas, including spontaneously arising gliomas without a predisposing germline alteration, which have addressed the associations of mutational load and dMMR. Retrospective series have shown disappointing results of patients with high TMB but without germline Lynch syndrome or MSI-high status treated with immune checkpoint blockers ⁷¹.

Other Biomarkers

It is possible that PD-1/PD-L1/TMB and others are bystanders and thus not the ideal biomarkers in a disease like GBM. Other indicators of immune engagement, such as inflammation markers, might be more valuable. One example may be assessment of the role of inflammasomes, which are important for CD8⁺ T-cell activation in response to PD-1 blockade. This can induce a PD-L1-NLRP3 inflammasome signaling cascade that ultimately may lead to the recruitment of

granulocytic myeloid-derived suppressor cells into tumor tissues ⁷². There may be other (yet unknown) and underexplored mechanisms dampening a potential anti-tumor immune response in GBM.

The blood-brain barrier

The blood-brain barrier (BBB) prevents the passive movement of cells and molecules that could induce inflammation or damage to the CNS. Endothelial cells, and the intact tight junctions between them, interact to maintain the integrity of the BBB, but do respond to environmental changes. Brain pathologies may permit the passage of circulating monocytes and lymphocytes from the periphery (57,58). Passage of T-cells across the BBB involves adhesion molecules and chemokines that might vary for different immune cell populations; e.g., CD8⁺ T-cells seem to be dependent on $\alpha 4$ integrin ⁷³.

PD-1 and PD-L1 antibodies are relatively large molecules and may not achieve high intratumoral concentrations, which could diminish their therapeutic benefit. However, they may not need to cross the BBB if they have a potent systemic immune effect. They may achieve some of their goals by acting on T-cells that are circulating or localized within lymphatic structures.

Various invasive and noninvasive approaches have been used for drug and gene delivery into the brain parenchyma with or without altering BBB homeostasis ^{74,75}. However, the macroscopically normal peritumoral zone is recognized as the primary site of GBM recurrence ⁷⁶. Immune surveillance, antigen presentation, and the role of myeloid cells in the CNS are complex and requires lymphatic drainage to lymph nodes ⁷⁷. Unlike extracranial organs, the brain contains a sparse lymphatic network. Cerebrospinal fluid is drained through the meningeal lymphatics into the deep cervical lymph nodes where brain-derived antigens and peripheral T-cells interact ⁷⁸. The vascular endothelial growth factor-C (VEGF-C; a growth factor for lymphatic vessels), can influence immune interactions via the BBB ⁷⁹. GL261 mice treated to increase VEGF-C expression were able to reject intracranial GBM tumor implants ⁷⁹. These mice also reject a re-challenge with flank tumor implants, demonstrating the generation of memory responses. The survival benefit in mice with only intracranial tumors treated with VEGF-C/anti-PD-1 combination therapy was similar to that in mice with both intracranial and flank tumors treated with checkpoint inhibitor therapy alone. Ligation of the deep cervical lymph nodes abrogated the VEGF-C benefit in mice with intracranial tumors, but not mice with both intracranial and flank implants. T-cell priming through

expression of VEGF-C in the cerebrospinal fluid, or through a flank tumor, enables checkpoint inhibition in the CNS. However, in these experiments, a tumor that was confined to the CNS at steady state (e.g., GBM), did not benefit from an immune checkpoint inhibitor alone ⁷⁹.

In contrast to GBM, combined anti-PD-1 and anti-CTLA-4 have extracranial and intracranial activity in melanoma and lung cancer⁸⁰. Mice with both a flank and an intracranial tumor respond better to combination immunotherapy than those with just intracranial melanoma ⁸¹. This may mean that immune checkpoint inhibitors are effective intracranially if the relevant immune signatures against the tumor are present to induce an extracranial immune response.

PD-1 Blockade in GBM: Ongoing Approaches

While the results of single-agent PD-1 blockade remain unsatisfactory to date, a number of new strategies are being considered, including the combination of PD-1 blockade with other immune checkpoint inhibitors, as well as targeted agents, radiation therapy, and chemotherapies.

Combination therapy with two or more therapeutic agents is a cornerstone of cancer therapy. There are barriers to the conduct of combination studies. These include toxicity, challenges with combination trial design, and the interpretation of endpoints. Endpoints, including the objective response rate, progression-free survival, and overall survival, have not evolved to optimally assess responses to immunotherapy. Trials are often underpowered and may require prolonged follow-up, often lasting multiple years⁸². Response assessment does not take into account the potential for immune checkpoint inhibitors to lead to either a delayed responses (e.g., continued tumor growth followed by tumor shrinkage) or “pseudoprogression” (e.g., apparent tumor growth driven by tumor inflammation followed by tumor shrinkage). It is yet to be seen if these issues will be addressed by the prospective use and validation of immune-related response criteria such as the Immunotherapy Response Assessment in Neuro-Oncology (iRANO)⁸³. Validation requires large datasets and iterative modifications and ultimately may be optimized for the immunotherapy setting.

A number of immunotherapies other than immune checkpoint blockers are currently under investigation alone, or in combination with PD-1 blockade. These include viral therapies, cancer vaccines, adoptive T-cells, and targeting ubiquitous viral antigens, such as those from cytomegalovirus (CMV), as well as therapies that aim to induce broad-based immune responses. These approaches may include targeting components of the innate immune system through oncolytic viruses, Toll-like receptors, the inflammasome, and other means to generate pathogen-associated molecular patterns and/or exploit self-molecules known as damage-associated molecular patterns to achieve immunogenic cell death^{84,85}.

Conclusions

While there is an urgent unmet need for glioma and GBM clinical research to find therapeutic breakthroughs, investment will need to continue in fundamental basic science in order to increase our understanding of the immunobiology of gliomas. More pre-clinical novel animal and humanized models are needed to better evaluate immune checkpoint inhibitors and combination immunotherapies (Table 4).

Phase I and phase II trials of PD-1/PD-L1 inhibitors, either alone or in combination, have reported safety and also some promising efficacy outcomes, but larger studies with adequate statistical power are needed.

Biomarkers for targeted therapies are usually based on a genetic aberration in the target itself (e.g., BRAF mutations, ALK translocations). These are reported clinically as either positive or negative assay results. This is different from immune biomarkers, which may need to be expressed as a matrix of PD-L1 expression, TMB, and tumor-derived interferon- γ gene signatures. While some have suggested developing an immunogram ⁸⁶ that includes all components of the immune system, identification of specific pathway drivers, such as TGF β , myeloid biology, WNT/ β -catenin, or altered tumor metabolism requires complex approaches, including metabolic, biochemical, cellular and nucleic acid-based assays.

Capitalizing on the success of PD-1 blockade in other malignancies, new approaches will need to build on new insights in glioma immunobiology to develop the next generation of clinical trials to help improve the outcome of people suffering from this devastating disease in a meaningful way.

Table 1; Examples of ongoing clinical trials including PD-1/ PD-L1 inhibitors in gliomas and glioblastoma

NCT Number	Study Title	Phase	Size (n)
NCT03426891	Pembrolizumab and Vorinostat Combined With Temozolomide for Newly Diagnosed Glioblastoma	1	32
NCT03491683	INO-5401 and INO-9012 Delivered by Electroporation (EP) in Combination With Cemiplimab (REGN2810) in Newly-Diagnosed Glioblastoma	1/ 2	52
NCT03532295	Epacadostat in Combination With Radiation Therapy and Avelumab in Patients With Recurrent Gliomas	1/ 2	55
NCT03665545	Pembrolizumab in Association With the IMA950/Poly-ICLC for Relapsing Glioblastoma	1/ 2	24
NCT02794883	Tremelimumab and Durvalumab in Combination or Alone in Treating Patients With Recurrent Malignant Glioma	2	36
NCT03493932	Cytokine Microdialysis for Real-Time Immune Monitoring in Glioblastoma Patients Undergoing Checkpoint Blockade	1	15
NCT03661723	Pembrolizumab and Reirradiation in Bevacizumab Naive and Bevacizumab Resistant Recurrent Glioblastoma	2	60
NCT03018288	Radiation Therapy Plus Temozolomide and Pembrolizumab With and Without HSPPC-96 in Newly Diagnosed Glioblastoma	2	108
NCT03557359	Nivolumab for Recurrent or Progressive IDH Mutant Gliomas	2	37
NCT03743662	Nivolumab With Radiation Therapy and Bevacizumab for Recurrent MGMT Methylated Glioblastoma	2	94
NCT03174197	Atezolizumab in Combination With Temozolomide and Radiation Therapy in Treating Patients With Newly Diagnosed Glioblastoma	1 /2	60
NCT03636477	A Study of Ad-RTS-hIL-12 With Velelimex in Combination With Nivolumab in Subjects With Glioblastoma; a Substudy to AT1001-102	1	18
NCT03233152	Intra-tumoral Ipilimumab Plus Intravenous Nivolumab Following the Resection of Recurrent Glioblastoma	1	6
NCT03722342	TTAC-0001 and Pembrolizumab Combination Trial in Recurrent Glioblastoma	1	20
NCT03047473	Avelumab in Patients With Newly Diagnosed Glioblastoma	2	30
NCT03707457	Biomarker-Driven Therapy Using Immune Activators With Nivolumab in Patients With First Recurrence of Glioblastoma	1	30
NCT03718767	Nivolumab in People With IDH-Mutant Gliomas With and Without Hypermutator Phenotype	2	95
NCT03341806	Avelumab With Laser Interstitial Therapy for Recurrent Glioblastoma	1	30
NCT04047706	Radiation, nivolumab, and BMS986205 (IDO inhibitor)	1	
NCT03367715	Nivolumab, Ipilimumab, and Short-course Radiotherapy in Adults With Newly Diagnosed, MGMT Unmethylated Glioblastoma	2	24
NCT03673787	A Trial of Ipatasertib in Combination With Atezolizumab	1/ 2	51
NCT02829931	Hypofractionated Stereotactic Irradiation With Nivolumab, Ipilimumab and Bevacizumab in Patients With Recurrent High Grade Gliomas	1	26
NCT03291314	Combination of Avelumab and Axitinib for the Treatment of Patients With Recurrent Glioblastoma	2	52
NCT03430791	Trial of Combination TTF(Optune), Nivolumab Plus/Minus Ipilimumab for Bevacizumab-naive, Recurrent Glioblastoma	2	60

Table 2; Examples of Completed Clinical Trials including PD-1/ PD-L1 inhibitors in gliomas and glioblastoma

NEWLY DIAGNOSED GLIOBLASTOMA TRIALS			
AGENTS	PHASE	TRIAL	COMMENTS
Nivolumab Compared to TMZ in patients with unmethylated MGMT promotor	3	CheckMate-498 NCT02617589	550 patients randomized This study confirmed the safety of PD-1 blockade plus radiotherapy for newly diagnosed GBM patients, but did not demonstrate either an overall survival or progression-free survival advantage ³
TMZ and RT with Nivolumab or Placebo, in patients with methylated MGMT promotor	3	CheckMate-548 NCT02667587	693 patients randomized The study did not demonstrate an improvement in progression-free survival, but its overall survival findings are pending further maturation of study follow-up data ³
RECURRENT GLIOBLASTOMA TRIALS			
AGENTS	PHASE	TRIAL	COMMENTS
Nivolumab versus bevacizumab	III	Chechmate-143 NCT02017717	<ul style="list-style-type: none"> • 369 patients randomized • No difference in overall outcome between the nivolumab or bevacizumab • Median OS was 9.8 months with nivolumab, 10.0 months with bevacizumab • Nivolumab responses were durable (median of 11 months), similarly to what is seen in other solid tumors and much longer than bevacizumab or other chemotherapies. • No steroid use and MGMT promoter methylation were associated with longer OS in the nivolumab arm versus the bevacizumab arm³
single-agent atezolizumab	I	NCT04047706	16 patients enrolled had received prior chemotherapy, and 50% prior bevacizumab. Ten patients (63%) experienced a treatment-related event. None was Grade 4–5 events ²⁹
bifunctional molecules targeting PD-L1 and TGF- β (bintrafusp alfa) ⁴⁰	IB	NCT02517398	35 patients enrolled Two patients had a PR, and 9 had SD The most common TRAEs were gingival bleeding (17.1%), asthenia (14.3%), pruritus, and rash (each 11.4%).

Abbreviations: MGMT: O⁶-methylguanine-DNA methyltransferase, PR: Partial Response, RT: Radiation Therapy, SD: Stable Disease, TMZ: Temozolomide, TRAE: Treatment Related Adverse Events,

Table 3; Examples of Completed Neoadjuvant Clinical Trials including PD-1/ PD-L1 inhibitors in glioblastoma

NEADJUVANT GLIOBLASTOMA TRIALS			
AGENTS	PHASE	TRIAL	COMMENTS
A Pre-surgical dose of nivolumab followed by post-surgical nivolumab	II	NCT02550249	A 30 patient single-arm trial ⁸⁷ . An increase in expression of chemokine transcripts, higher immune cell infiltration, and augmented T-cell receptor clonal diversity among tumor-infiltrating T lymphocytes, was reported in patients receiving nivolumab compared to those who did not.
Pre- and post-operative pembrolizumab versus post-operative therapy alone	II		35 patient enrolled Better survival in the presurgical-surgical PD-1 ⁸⁸ , together with an upregulation of T-cell and interferon- γ -related gene expression, but downregulation of cell cycle-related gene expression within the tumor. This was not seen in the control group. Peripheral markers of immune engagement were observed more often in the neoadjuvant group than in patients treated only in the post-operative setting.
pre- and post-operative pembrolizumab	II	NCT02337686	15 patients enrolled Indirect signs of immune engagement was observed. The study concluded that anti-PD-1 monotherapy is insufficient for an immunomodulatory response in the majority of GBM patients, likely due to paucity of T-cells within the tumor microenvironment and a CD68 ⁺ macrophage preponderance ⁸⁹

Table 4; Summary of the issues, barriers, recommendations and future directions

Why PD-1 inhibitors were successful in many cancers but not in GBM? The Major Biological Barriers
<ul style="list-style-type: none"> ▪ Tumor heterogeneity ▪ Drug delivery, blood brain barrier issues and the challenges with obtaining brain biopsies ▪ Immunosuppression ▪ Lack of neoantigens and low tumor mutational burden
Challenges in Identifying Reliable Immune Biomarkers in GBM
<ul style="list-style-type: none"> ▪ Unless there is a confirmed effective immunotherapy in GBM it will not be possible to validate relevant biomarkers. ▪ Most biomarkers are discovered in reverse. They may represent association and not causation; i.e., tumor mutational burden (TMB) and other usual biomarkers might not have the same relevance in GBM and other cancers. ▪ Currently used biomarkers for PD-1/PD-L1 inhibitors include PD-L1 expression, TMB, TIL infiltration, and immune activation gene expression profiles. ▪ PD-L1 expression may also change over time. Laboratory evaluation is typically based on archival specimens, which may not reflect the phenotype at the time of PD-1 administration. ▪ TMB may be a useful predictor in some solid tumors, but it has not been evaluated specifically in GBM. ▪ Large sample sizes and large validation sets are required to discover reliable biomarkers. This has been a challenge in GBM. ▪ There is a need to explore other biomarkers in addition to the traditional PD-L1, TMB, and others that might be helpful in other cancers but not in GBM.
Recommendations and Future Directions
<ul style="list-style-type: none"> ▪ Design trials that can answer questions, minimize risk of producing uninterpretable results ▪ Minimize use of corticosteroids ▪ Develop strategies that can address heterogeneity ▪ Encourage neoadjuvant/phase 0/window of opportunity studies ▪ Develop more useful biomarkers/T-cell assays, etc. ▪ Address drug delivery issues

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